THE RELATIONSHIP BETWEEN SEASONAL STEROID HORMONE CONCENTRATIONS AND THE REPRODUCTIVE CYCLE IN THE NORTHERN PACIFIC RATTLESNAKE, *CROTALUS OREGANUS*

A Thesis

Presented to

The Faculty of California Polytechnic State University

San Luis Obispo

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in Biological Sciences

By

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June, 2009

TITLE:	The Relationship Between Plasma Steroid Hormone Concentrations and the Reproductive Cycle in the Northern Pacific Rattlesnake, *Crotalus oreganus*
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ABSTRACT

The Relationship Between Plasma Steroid Hormone Concentrations and the Reproductive Cycle in the Northern Pacific Rattlesnake, *Crotalus oreganus*

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To gain a better understanding of the role of steroid hormones in vertebrate reproduction, we quantified steroid hormone concentrations in a free ranging population of the Northern Pacific rattlesnake, *Crotalus oreganus*. Plasma steroid hormone concentrations were quantified for both male and female snakes throughout the active season (Mar-Oct). We measured testosterone (T), 5α-dihydrotestosterone (DHT), and corticosterone (B) concentrations in male and female snakes. 17β-estradiol (E2) and progesterone (P) were measured in females only. We also observed breeding behaviors (e.g. consortship, courtship, and copulation) in the field and measured testis and follicle size in male and female snakes from museum collections. Our results indicate that *C. oreganus* in central California utilizes a bimodal pattern of breeding, with mating and agonistic behavior occurring in the spring and the late summer/fall. Each breeding season corresponds with elevated or highly variable androgen (T and DHT) levels. Several female snakes had high E2 concentrations in the spring and fall, coincident with vitellogenesis and mating. Females with high E2 concentrations also had high T and DHT concentrations. Corticosterone concentrations in males are not related to either time of year or concentrations of any other hormones quantified. This suggests that the breeding season in this population may not demand a significant increase in energy mobilization by glucocorticoids. Measurements of testis volume show that testes are regressed in the spring when the majority of breeding was observed in this population and reach peak volume in August and September during spermatogenesis. Multiple regression analyses revealed that in female snakes, P is positively correlated with T and DHT, and E2 is correlated with T. Since these results are strictly descriptive, experimental studies are needed to identify the functional significance of these results.

Keywords: hormone, testosterone, dihydrotestosterone, corticosterone, estradiol, progesterone, reptile, snake, reproduction

ACKNOWLEDGEMENTS

I would like to acknowledge several people for assisting me in the compilation of this thesis. First I would like to thank my advisor and committee chair, Dr. Emily Taylor, for all of her advice and guidance throughout this project. I would also like to acknowledge Dr. Gita Kolluru, Dr. Chad Montgomery, Dr. John Perrine, Dr. Jeff Sklar, and Dr. Christine Strand for comments on drafts of this document. And lastly, I would like to acknowledge Jordan Ahle, Marty Feldner, Tony Frazier, Peter Jackson-Tooby, Lea Kromschroeder, and Bree Putman for assistance in data collection in the field. This project would not have been possible without the help of these individuals.

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1. Introduction

Pit vipers inhabiting the Temperate Zone have recently emerged as model organisms for investigation of vertebrate reproductive physiology. As is the case for most vertebrates, the reproductive cycles of snakes are mediated by steroid hormones (reviewed in Taylor and DeNardo, in press). The roles of these hormones in snakes are complex and are still not fully understood. There is a large body of experimental research on the role of hormones in the reproductive cycles of snakes. However, research in this area is dominated by one subspecies of garter snake, *Thamnophis sirtalis parietalis* (reviewed in Taylor and DeNardo, in press). These studies have revealed much about the role of hormones in this species. However, the roles that steroid hormones play in reproductive systems are not ubiquitous across all snake groups. Correlational studies relating plasma steroid hormone concentrations to events of the reproductive cycle (i.e. vitellogenesis, spermatogenesis, and mating) of vipers have revealed differences between the hormonal patterns of temperate vipers and those of *T. s. parietalis* (see Almeida-Santos et al., 2004, Camazine et al., 1980; Crews, 1984; Graham et al., 2008; Schuett et al., 1997; 2002; 2005; 2006). Is this the only variation? Are there other differences among studies that justify the idea that these things are complex and not ubiquitous? The role of hormones in viper reproductive systems requires further investigation, as these snakes have a unique ecology and physiology which are very different from that of *T. s. parietalis*. Research on pit vipers with contrasting mating systems will likely be fruitful in determining the roles of these hormones in vertebrate reproductive cycles.

Here I review the mating systems of temperate pit vipers and the role of steroid hormones within these systems. In this chapter, I also describe what is known about the

reproductive cycle of the Northern Pacific rattlesnake, *Crotalus oreganus*. Chapter 2 is presented in manuscript form and reports the results of my studies on the relationship between hormones and reproduction in this species.

2. *Mating systems*

The mating patterns of pit vipers are complex. They can vary within family, genus, and even species (reviewed in Aldridge and Duvall, 2002). Such diversity is possible due to the fact that, in most snakes, reproductive behaviors are not restricted to the time of ovulation. Female snakes can store sperm from the breeding season until the time of ovulation. In some species, females are able to store sperm for several months (long term sperm storage, LTSS); others store sperm for only a few weeks (short term sperm storage, STSS, Schuett et al., 1992). In all temperate vipers studied, ovulation occurs in the early summer and parturition occurs in the fall (reviewed in Aldridge and Duvall, 2002). Ovulation and mating generally take place at the time of year that will result in offspring being birthed at a time that optimizes survival.

The evolution of sperm storage mechanisms in snakes has relieved them from the need to breed at the time of ovulation in early summer and has led to the emergence of three mating patterns among temperate zone pit vipers: (1) Spring breeding only (spring unimodal), (2) late summer and fall breeding (fall unimodal), and (3) breeding in both seasons (bimodal). Snakes utilizing these different patterns have contrasting physiological cycles (e.g. hormone and gonadal cycles). Gonadal cycles have been used to describe the mating systems of many pit viper species (*Agkistrodon piscivorus*:

Johnson et al., 1982; *C. atrox*: Tinkle, 1962; *C. helleri*: Aldridge, 2002; *C. horridus*: Gibbons, 1972: *C. lepidus*: Goldberg, 2000a; *C. mitchellii*: Goldberg, 2000b; *Crotalus molossus*: Goldberg, 1999a; *C. oreganus*: Aldridge, 2002; *C. pricei*: Goldberg, 2000c; *C. ruber*: Goldberg, 1999b; *C. scutulatus*: Goldberg and Rosen, 2000; *C. tigris*: Goldberg, 2000d; *C. viridis*: Aldridge, 1979; *C. willardi*: Holycross and Goldberg, 2001). Recent studies have investigated sex steroid hormone concentrations of species with known mating systems in an effort to correlate steroid hormone concentrations with events of the reproductive cycle (Almeida-Santos et al., 2004; Graham et al., 2008; Johnson et al., 1982; Schuett et al., 1997; 2002; 2005; 2006; Taylor et al., 2004; Zaidan et al, 2003). These studies are summarized in the sections below.

2.2 Male Cycles

Only two species exhibit the spring unimodal pattern (*C. mitchellii* and *C. ruber*, Aldridge and Duvall, 2002). In these snakes, female LTSS apparently does not take place. In all temperate pit vipers studied, spermatogenesis takes place in the late summer and fall before over-wintering (reviewed in Aldridge and Duvall, 2002). Therefore, male snakes that breed in the spring must store sperm in the vas deferens over winter until the spring breeding season (Schuett, 1992). Spring unimodal species are the only pit vipers where spermatogenesis is completely uncoupled from mating. Neither of these species has been the subject of extensive study on reproduction. Further study of this mating system is necessary, as these species provide the opportunity to study the hormonal

regulation of spermatogenesis, because breeding behavior and spermatogenesis are completely dissociated.

A single breeding season in the late summer and fall coincident with spermatogenesis is much more common among temperate vipers (Aldridge and Duvall, 2002). In these species, LTSS by females is obligatory (Schuett, 1992). This strategy is exhibited by *A. piscivorus, C. adamanteus, C. concolor, C. horridus* (eastern populations), *C. molossus, C. oreganus* (northern populations), *C . tigris, C. viridis, C. willardi,* and *Sistrurus miliarus* (reviewed in Aldridge and Duvall, 2002).

Bimodal breeding patterns, in which snakes mate in spring and fall, are also common in temperate vipers. Bimodal patterns are found in *Agkistrodon contortrix, C. atrox, C. cerastes, C. horridus* (western populations), *C. oreganus* (southern populations*), C. helleri, C. scutulatus,* and *S. catenatus* (Aldridge and Duvall, 2002). In the bimodal pattern, mating can either be associated with spermatogenesis (fall) or dissociated from spermatogenesis (spring). These species exhibit facultative LTSS, meaning that a female can either use sperm from a fall mating that was stored over winter, or sperm from a spring mating event, or both. Schuett and Gillingham (1986) found that *A. contortrix*females can use stored sperm from the fall mating season and sperm from the spring breeding season to fertilize a single litter in summer. The factors determining which sperm (i.e., sperm from fall copulation or from spring copulation) are used to fertilize eggs at the time of ovulation in the summer are not understood.

2.3 Female Cycles

Female viper species, in addition to differing in the timing of mating, differ in the timing of vitellogenesis (i.e., production of the protein vitellogenin, which stimulates follicular yolk deposition). Only one temperate pit viper studied, *C. atrox,* initiates vitellogenesis in the spring (Type I vitellogenesis, Aldridge, 1979; Taylor et al., 2004). All other species studied begin vitellogenesis in the summer or fall, pause follicular activity over winter, and resume and complete vitellogenesis in the spring and early summer (Type II vitellogenesis, Aldridge, 1979; Aldridge and Duvall, 2002). The timing of other events in the reproductive cycle (e.g. ovulation, and parturition) is conserved, with females ovulating in early summer and giving birth to live young in late summer (reviewed in Aldridge and Duvall, 2002).

3. Steroid hormones as proximate mediators of pit viper mating systems

The role of steroid hormones in the reptilian reproductive cycle has been intensely studied. Androgens (testosterone, T, and 5α-dihydrotestosterone, DHT), are involved in male sexual behavior and gametogenesis in most taxa (Norris, 1997; Whittier and Tokarz, 1992a). Progesterone (P) and 17β-estradiol (E2) are produced by ovarian follicles and the corpus luteum in female vertebrates and have diverse roles in mediating reproductive behavior and other events of reproduction (e.g. vitellogenesis and gestation, Callard et al., 1990a; 1990b; Custodia-Lora and Callard, 2002). Glucocorticoids are used to mobilize stored energy reserves during stressful events and have recently been implicated as

players in the reproductive cycles of birds and reptiles (reviewed in Romero, 2002; Moore and Jessop, 2003).

Not until recently (1980's) have the relationships between sex steroid hormones and the reproductive cycles of snakes been studied. Pit vipers have become model organisms for studying these hormones in wild populations (Schuett et al., 2006) because they are common, easily studied in the wild, and long-lived. The majority of this research has focused on male androgen cycles. Only one study (Taylor et al., 2004) has quantified female steroid hormone concentrations in free ranging temperate pit vipers. In the sections that follow (3.1-3.4) I discuss what is known about the role of hormones in snake reproduction and then summarize what has been learned from studying free ranging vipers.

3.1 Androgens

The androgens T and DHT stimulate mating behavior in male reptiles (Whittier and Tokarz, 1992a). In many vertebrates mating and agonistic behavior (i.e., male-male fighting) are associated with gonadal activity (associated breeding, Crews and Moore, 1986). Steroid hormones are key mediators of gamete maturation in vertebrates (reviewed in McLachlan et al., 1996). Consequently this makes them convenient mediators of reproductive behaviors, because in associated breeders mating and gonadal activity are coupled. However, in species that utilize sperm storage, the act of mating and gonadal activity of both males and females can be separated by months (dissociated breeding, Crews and Moore, 1986). In these species, androgens may not play as large of a role in

inducing mating behavior, as the gonads are often regressed at the time of mating. However, the results of studies investigating this phenomenon in snakes have been mixed.

The red sided garter snake, *T. s. parietalis*, has historically been the model organism demonstrating dissociated breeding. The population most heavily studied is located in Manitoba, Canada. Males of this species mate during spring, when circulating T levels are low and their testes are regressed (Camazine et al., 1980). The primary stimulus for breeding behavior in *T. s. parietalis* is temperature. These snakes must be held at low temperatures over winter and then warmed in order to elicit breeding behavior (Hawley and Aleksuik, 1975). *Thamnophis s. parietalis* will display mating behavior and court females under these conditions even after castration or hypophysectomy (Camazine et al., 1980). The breeding season is also not prolonged by androgen supplementation. These experiments provide strong evidence that elevated androgen concentrations are not needed at the time of mating in this species. However, studies on this same population and closely related subspecies have found that plasma androgens are actually quite high in the spring breeding season (Krohmer et al., 1987; Weil, 1985). Saint Girons et al. (1993) suggest that the findings of Crews et al. (1984) may have been due to laboratory methods that increased metabolic clearance of androgens just before hibernation. He suggests that plasma androgens may remain high throughout winter due to low rates of metabolic clearance at low temperatures. Saint Girons et al. (1993), however, agrees with Crews (1991) that androgens may be necessary only to condition male sexual behavior, and that high androgen levels are not obligatory at the time of copulation in snakes. Descriptive studies on vipers (see 3.5) reveal that androgens are elevated in the spring at

the time of mating, but this condition may not be required for snakes to mate (St. Girons et al., 1993).

The roles of androgens in the female reproductive cycle are poorly understood (Staub and De Beer, 1997). Testosterone is elevated during vitellogenesis in several snake species studied (Bona Gallo et al., 1980, Taylor et al., 2004). This could be because E2 is derived from T via aromatization. Experimental studies have yet to investigate this phenomenon. Testosterone and DHT have also been implicated in the stimulation of female estrous behavior and receptivity in vertebrates (Staub and De Beer, 1997; Whittier and Tokarz, 1992b). It is clear that these hormones do play some role in the reproductive cycles of female snakes, but the exact role has yet to be elucidated (Staub and De Beer, 1997).

3.2 Estradiol and progesterone

Estradiol is produced by granulosa cells in ovarian follicles and is thought to be the primary stimulus for both estrous behavior and synthesis of yolk proteins during vitellogenesis in female reptiles (Whittier and Tokarz, 1992b, Callard et al., 1990a). Vitellogenesis occurs in both viviparous and oviparous vertebrates. It is the process of synthesis and deposition of yolk proteins in the developing follicle. In squamate reptiles, E2 directly stimulates the synthesis of yolk proteins by the liver (Callard et al., 1990a). However, studies quantifying E2 concentrations during vitellogenesis in snakes have not yielded consistent results. Studies have found that E2 concentrations are independent of vitellogenic state, suggesting some regulatory ability with respect to E2 receptivity of

vitellogenin-producing hepatocytes, allowing snakes to have high circulating E2 and not enter vitellogenesis (Bonnet et al., 1994). More research is required in this area.

Another steroid hormone, P, suppresses vitellogenesis and maintains gravidity or pregnancy (Callard et al., 1990b). Progesterone is thought to contribute to the reduction in yolk deposition observed in some vertebrates and has been implicated as a proximate factor in the evolution of viviparity in reptiles and mammals (Callard et al, 1990b; Guillette, 1993). Progesterone is produced by granulosa cells in the ovarian follicle and the corpus luteum in reproductive female reptiles (Klicka and Mahmoud, 1977), and by the adrenal glands in male and non-reproductive female snakes (e.g. *Thamnophis*, Highfill and Mead, 1975). In the aspic viper (*Vipera aspis*), P concentrations are low in the follicular phase (vitellogenesis), and rise during the luteal (post-ovulation) phase until parturition (Bonnet et al., 2001). This supports the hypothesis that P is an antagonist of E2 and acts to redirect hepatocytes away from synthesis of vitellogenic proteins and toward other functions (Bonnet et al., 2001).

Progesterone also plays a role in maintaining pregnancy. This function has been well established in reptiles. Progesterone decreases myometrial activity and prolongs gravidity leading to egg retention in the painted turtle, *Chrysemys picta*, implicating P as a key player in the maintenance of pregnancy or gravidity in reptiles (Callard and Hirsch, 1976). However, studies of viviparous snakes reveal that exogenous administration of P has no effect on gestation length, suggesting some fetal control of parturition (Bonnet et al., 2001). It appears that P is directly involved in the maintenance of pregnancy in reptiles; however, a drop in P concentrations may not be the primary stimulus for

parturition in all species (Bonnet et al., 2001, Callard and Hirsch., 1976; Taylor et al., 2004).

3.3 Corticosterone

Corticosterone, a glucocorticoid, is not typically considered a sex steroid hormone. However, most reptiles experience peak B concentrations during the breeding season (reviewed by Romero, 2002). One hypothesis for this phenomenon is that, because peak B concentrations occur at the most energy-limited time of year, B acts as an energy mobilizer to help the individual cope with limited resources and/or increased energy expenditure. This hypothesis has been coined the "energy mobilization hypothesis" (EMH) (Romero, 2002). Reproduction is costly for most reptiles, especially for females (reviewed in Shine, 1980). Female reptiles bear the burden of carrying and providing energy for offspring. Under the EMH, the periods of vitellogenesis and gestation are likely to be the most energy-limited times of the year and, thus, coincide with peak B levels. The breeding season can also be energetically costly for males because they engage in mate searching, male-male combat, androgen production, and spermatogenesis (Chandola et al., 1974; Olsson et al., 1997). Assuming prey availability is uniform across the season, these events of the male and female cycle should coincide with peak B levels under the EMH. This is the case for most reptiles. However, the two pit vipers studied to date do not follow this pattern (see sec. 3.4).

3.4. Hormone cycles in pit vipers

Temperate vipers are model organisms for the studying the relationship between steroid hormones and reproduction in wild populations**.** They are large-bodied, present in large numbers in many parts of their range, and utilize diverse mating strategies (Almeida-Santos et al., 2004; Beaupre and Duvall, 1998; Schuett et al., 2006). Although these animals have many characteristics that make them ideal candidates for experimental investigation of hormone action in vertebrates, the role of hormones in sexual behavior has not been studied experimentally in any pit vipers. However, correlational studies on several species have revealed much about the roles that steroid hormones play in the ecology and physiology of free-ranging vertebrates.

Testosterone and DHT are elevated at the time of both breeding and spermatogenesis in all male vipers studied to date (*A. contortrix*: Schuett et al., 1997; *A. piscivorous*: Graham et al., 2008; *C. atrox*: Taylor et al., 2004; *C. molossus*: Schuett et al., 2005; *C. scutulatus*: Schuett et al., 2002; *V. aspis*: Saint Girons et al., 1993). Of these, only *C. molossus* and *A. piscivorous* breed unimodally in the late summer and fall. The androgen profile for these species has a single peak in late summer coincident with spermatogenesis and mating (Graham et al., 2008; Johnson et al., 1982; Schuett et al., 2005). All other pit vipers studied exhibit the bimodal pattern, with two androgen peaks, one in spring (dissociated from spermatogenesis) and one in the late summer/fall (associated with spermatogenesis). In *C. atrox*, T is at peak concentrations early in the spring breeding season (March) and during an additional breeding season and peak spermatogenesis (August) (Taylor et al., 2004). However, most matings occurred in

April, when T concentrations, while elevated, were lower than in March. It is unclear why T concentrations are higher in March than in April, when the breeding season is at its peak. This trend has been observed in temperate garter snakes as well (Moore et al., 2000). This supports the organizational hypothesis, under which androgens condition male snakes for reproduction at a slightly later date (Crews, 1991, Saint Girons, 1993). Basal androgen levels are recorded in May and June in all bimodal pit vipers studied.

Schuett et al. (2006) quantified plasma T concentrations in *C. atrox* in the Sonoran Desert basking on warm winter days. They found that plasma T remains above baseline levels throughout the winter. Whether this phenomenon is due to a low metabolic clearance of T produced in the fall or a sustained production throughout the winter is not clear. Measurement of androgen or other hormone concentrations during winter have not been possible in other species because snakes remain underground in hibernacula.

Steroid hormone concentrations have been quantified in female pit vipers from two populations (*C. durissus*, Almeida-Santos et al., 2004; *C. atrox*, Taylor et al., 2004). These studies focused on the hormones E2 and P. 17β-estadiol is elevated during secondary vitellogenesis in both species. Progesterone concentrations were basal at this time, which supports the hypothesis that P and E2 may be antagonists. Pregnancy profiles in these two species are similar, with peak P concentrations recorded early in gestation followed by a steady decline until parturition. 17β-estradiol is basal during pregnancy in both species. *Crotalus atrox* in the Sonoran Desert displays a vitellogenic pattern very different from most rattlesnakes. Most rattlesnakes initiate secondary vitellogenesis in the fall before a reproductive year (reviewed in Aldridge and Duvall, 2002). This cycle

would presumably coincide with elevated E2 levels in both the fall and spring. *Crotalus atrox* in the Sonoran Desert utilize Type I vitellogenesis and have a unimodal E2 profile with basal levels recorded during fall breeding season. The proximate cue for breeding behavior during the fall breeding season is unknown. To date, an E2 profile has never been recorded for a female pit viper that initiates vitellogenesis in the fall.

Corticosterone levels in free-ranging male pit vipers have been quantified in *C. atrox* (Taylor et al., 2004) and *A. piscivorus* (Graham et al., 2008). Results for free ranging male snakes in these studies indicate that B is not elevated during the breeding season, even though male pit vipers tend to exhibit extensive mate-searching forays during the breeding season(s). This may be due to the fact that male vipers generally have large body fat reserves and low metabolic rates that may allow them to get through periods of increased activity without launching an adrenal response (Andrews and Pough, 1985; Taylor et al, 2004). *Agkistrodon piscivorus*, however, shows a seasonal trend in B concentrations, but the peak is not associated with reproduction. Peak B is recorded just before the breeding season in April and May. Graham et al. (2008) speculate that this could be due to increased basking and foraging behavior or the initiation of spermatogenesis.

Taylor et al. (2004) reported B concentrations in reproductive and non-reproductive female *C. atrox.* Non-reproductive females showed no trend in B concentrations throughout the season. Corticosterone concentrations in reproductive snakes rose steadily throughout gestation and peaked immediately prior to parturition. The authors speculated that B may play a role in stimulating parturition. Controlled experiments are required to determine any causal role of B in reptile parturition.

4. Anatomical correlates of reproduction

Most of what is known about the diversity of mating cycles in temperate zone pit vipers has been derived from histological examination of reproductive tissues. Histological analysis of both fresh tissue and preserved tissue from museum collections are widely used in determining the seasonality of reproductive events (e.g. spermatogenesis, vitellogenesis, mating, parturition). Cross sections of the testes of male snakes followed by histological examination can be used to determine the seasonal cycle of spermatogenesis. Examination of female follicles throughout the year, either by palpation or dissection, can provide researchers with timelines for vitellogenesis.

Male squamate reptiles also have a reproductive tissue that is absent in mammals, the sexual segment of the kidney (SSK). Secretions from the SSK provide sustenance for mature sperm (Cuellar et al., 1972). The SSK is an indicator of sexual activity in male reptiles, because it becomes hypertrophied in response to testosterone (Prasad and Sanyal, 1969). The response of the SSK to elevated T levels allows researchers to determine the time of year in which T is elevated.

Histological studies have confirmed that spermatogenesis is restricted to the summer and early fall in all temperate pit vipers (reviewed in Aldridge and Duvall, 2002). Histological studies of the SSK have revealed two patterns of SSK hypertrophy. First, fall unimodal snakes exhibit SSK hypertrophy in the late summer and fall coincident with spermatogenesis and mating (Graham et al, 2008; Johnson et al., 1982). Second, bimodal species exhibit SSK hypertrophy in the spring and late summer/fall

(Aldridge, 2002). Spring unimodal snakes are expected to follow the same pattern, because T should be elevated during fall spermatogenesis and the spring breeding season. However, the SSK has never been studied in these species.

Several studies have used testis mass and/or length to assess reproductive condition in pit vipers (Graham et al., 2008; Johnson et al., 1982; Schuett et al., 2002). Of these studies, all but one were conducted on snakes with a unimodal summer/fall breeding cycle (e.g., *A. piscivorus*, Graham et al., 2008; Johnson et al., 1982). In this species, testis mass and length were lowest in early spring and increase throughout spermatogenesis (Graham et al, 2008, Johnson et al., 1982). The only study measuring testis mass and or length in a bimodal pit viper was conducted on *C. scutulatus* (Schuett et al., 2002). This study found that testis length and mass were significantly elevated in the spring breeding season, coincident with mating and elevated T concentration, and in the late summer and fall, coincident with spermatogenesis, mating, and elevated T concentrations. Studies on other reptiles including snakes, turtles, and alligators, have shown that elevated androgen concentrations and breeding behavior do not always coincide with testis recrudescence. In the American Alligator, *Alligator mississippiensis*, testicular mass does not increase when plasma T concentrations increase in the late summer breeding season (Lance, 1989). In the softshell turtle, *Trionyx sinensis*, and the red-sided gartersnake, *T. s. parietalis*, spring breeding is coincident with low or variable T concentrations and regressed testes (Camazine et al., 1980; Licht, 1982). These studies suggest that the breeding season and androgen production can be temporally separated from testis recrudescence. However, too few studies on testis size in bimodal snakes with known steroid hormone profiles are available for further evaluation of this phenomenon.

Further investigation into the relationship between the breeding season and testis size is required to ascertain the extent to which testis size is a reliable indicator of reproductive activity and/or androgen levels.

5. Reproduction in the Northern Pacific rattlesnake (*Crotalus oreganus*)

The Northern Pacific rattlesnake, *C. oreganus*, is a member of the western rattlesnake group. This group was previously considered one species with nine subspecies and has recently been split into seven species, including *C. oreganus* (Ashton and de Queiroz, 2001; Douglas et al., 2002). The range of *C. oreganus* extends from central California to British Columbia (Stebbins, 2003).

Several studies of the ecology and natural history of *C. oreganus* have been conducted in the northern portion of this species' range (Diller and Wallace, 1984; 2002; Macartney and Gregory, 1988). These studies have shed light on life history traits such as fecundity, growth rate, age at maturity, and mating system in northern populations. Fitch (1949) studied these traits in a population of *C. oreganus* in central California, which is the southern end of this species' range. These studies have revealed great variation in life history traits between northern and southern populations. Studies on geographic variation in life history traits within a species are extremely informative because they minimize potentially confounding phylogenetic effects (Macartney and Gregory, 1988). This makes *C. oreganus* a good model for any study investigating environmental influences on the reproductive physiology of vertebrates.

5.1 Reproduction in Crotalus oreganus

Crotalus oreganus, like most pit vipers, breed on a less-than-annual basis. However, less-than-annual reproduction is resource-based, and annual reproduction has been observed in females of this species, presumably when resources are sufficiently available (Diller and Wallace, 2002). The frequency of breeding differs from population to population. Populations in British Columbia breed, at most, biennially and often triennially (Macartney and Gregory, 1988). Populations in Idaho and California can breed either annually, biennially, or triennially (Diller and Wallace, 1984; Fitch, 1949). Litter size estimates for this species range from 4.6 in northern populations (Macartney and Gregory, 1988) to 9.9 in central California (Fitch, 1949). The active season of snakes in the northern regions of the range of *C. oreganus* is significantly shorter than that of southern populations in California. The higher estimate of litter size in southern populations may be due to increased time to build fat reserves in the fall before a reproductive year. It likely also results from differences in methods, as Fitch's (1949) estimate was based on counts of vitellogenic follicles in the spring, and estimates for northern populations (Macartney and Gregory, 1988) are based on counts of offspring birthed. The litter size of vipers may be influenced by food intake during vitellogenesis, and counts of spring follicles are certainly overestimates of fecundity (Lourdais et al., 2003).

There are also intraspecific differences in the timing of the breeding season in *C. oreganus*. California populations display a bimodal breeding pattern, with mating in both spring and fall (Aldridge, 2002; Hersek et al., 1992; see Chapter 2). This allows males to

mate in either an associated (late summer/fall) or dissociated (spring) manner. This tactic requires that both males and females be able to store sperm from the fall breeding season until mating in the spring and ovulation in the summer (Schuett, 1992). Northern populations display a different seasonal pattern of reproduction. Macartney and Gregory (1988) found that *C. oreganus* in British Columbia mate in the late summer only (unimodal). Diller and Wallace (1984) described a similar pattern for populations in northern Idaho. In these populations, LTSS by females is obligate. It is unclear where geographically this species makes the transition from unimodal to bimodal breeding. Populations of *C. oreganus* between the San Francisco Bay Area and Idaho have not been studied. Future studies in these areas would be informative in discerning the environmental parameters conducive to each breeding strategy.

There also may be variation in the timing of vitellogenesis within *C. oreganus*. Both the Idaho and British Columbia populations initiate vitellogenesis in the late summer or fall (Diller and Wallace, 1984; Macartney and Gregory, 1988). Vitellogenesis is then completed upon emergence from hibernacula. Fitch (1949) determined that vitellogenesis is restricted to the spring in California. However, a comparison of Fitch's data to that of Rahn's (1942) study on a closely related species, *C. viridis*, indicate that Fitch's data are not necessarily indicative of spring-only vitellogenesis (see Aldridge and Duvall, 2002). All other rattlesnakes studied to date, with the exception of a population of *C. atrox*, initiate vitellogenesis before the winter of a reproductive year (reviewed in Aldridge and Duvall, 2002).

In summary, *C. oreganus* exhibits substantial geographic variation in reproductive strategies in both sexes. The large range of this species, specifically its large latitudinal

distribution, may result in a multitude of selection pressures leading to divergence in reproductive strategies within this species. Fecundity and the frequency of reproduction vary with latitude (Diller and Wallace, 2002; Fitch, 1949; Macartney and Gregory, 1988). There are also distinct differences in the timing of mating among populations of this species. Northern populations follow the unimodal pattern, and southern populations follow the bimodal pattern. These regions may also differ in the timing of reproductive investment by females (i.e. vitellogenesis), although the data supporting this are controversial. It is not known if these latitudinal transitions in the breeding cycle represent a distinct break in reproductive tactic at a certain latitude driven by environmental variables (e.g. length of the active season), or if these characteristics are two extremes along a latitudinal gradient. Future study of populations inhabiting diverse environments would be valuable in determining the ultimate forces at work in the diversification of the mating patterns in *C. oreganus*.

CHAPTER II:

1. Introduction

The roles of hormones in the mating systems of New World pit vipers (family Viperidae, subfamily Crotalinae) and snakes in general are poorly understood. The majority of research in this area has focused on one subspecies of garter snake, *Thamnophis sirtalis parietalis* (reviewed in Taylor and DeNardo, in press). Garter snakes have an ecology and physiology that are different from that of pit vipers. Consequently, the roles of steroid hormones in the two snake groups are likely to be different. Crotaline snakes have been the subject of several recent studies that have contributed to the understanding of the role of steroid hormones in snake reproductive systems (Almeida-Santos et al., 2004; Graham et al., 2008; Johnson et al., 1982; Taylor et al., 2004; Schuett et al., 1997; 2002; 2005). However, these works are preliminary, and require further investigation of a broad range of hormones in both sexes if an understanding of the relationship between steroid hormones and the reproductive cycle of free-ranging vertebrates is ever to be achieved.

Crotaline snakes are large-bodied, and have diverse mating systems, which make them ideal model organisms for studying the ecology and physiology of vertebrate reproduction (Beaupre and Duvall, 1998; Almeida-Santos et al., 2004; Schuett et al., 2006). These characteristics have allowed researchers to examine the relationship between steroid hormone concentrations and reproduction in several temperate pit viper species including copperheads (*Agkistrodon contortrix*, Schuett et al., 1997), cottonmouths (*A. piscivorus*, Graham et al., 2008; Zaidan III et al., 2003), western

diamond-backed rattlesnakes; (*Crotalus atrox*, Taylor et al., 2004), black-tailed rattlesnakes (*C. molossus*, Schuett et al., 2005), and Mojave rattlesnakes (*C. scutulatus*, Schuett et al., 2002). However, this research has focused heavily on the role of male androgens, and, with the exception of Taylor et al. (2004), has ignored females.

Studies of temperate pit vipers have revealed two major patterns in mating systems among these species, bimodal and unimodal breeding. Many rattlesnakes show a bimodal pattern of breeding with mating taking place in the spring and late summer (reviewed in Aldridge and Duvall, 2002). Others, including some populations of the northern Pacific rattlesnake (*C. oreganus*), have a single breeding season in the late summer (Macartney and Gregory, 1988). Snakes that breed exclusively in the late summer or early fall are said to follow an associated breeding pattern, where breeding behavior is associated with male gonadal activity (Saint Girons, 1982), as all temperate zone pit vipers studied to date undergo spermatogenesis in the late summer/fall (Aldridge and Duvall, 2002). However, in snakes displaying the bimodal pattern, the spring breeding season is dissociated from spermatogenesis. The dissociation of breeding behaviors from spermatogenesis in bimodally breeding rattlesnakes has allowed for examination of the role of hormones in male snakes during the spring breeding season independent of spermatogenesis (St. Girons et al., 1993). Studies investigating this phenomenon in reptiles have yielded mixed results. In some species the spring breeding season coincides with elevated androgen levels and/or testicular hypertrophy (Schuett et al., 2005; Taylor et al., 2004). In others, testes are regressed and androgen concentrations are low or variable (Camazine et al., 1980; Licht, 1989).

In addition to variation in the breeding cycle, there is also variation in the timing of vitellogenesis in female vipers. Most rattlesnakes initiate vitellogenesis in the fall, followed by a pause in follicular activity over winter, and a continuation and completion of vitellogenesis by the time of ovulation the following spring/summer (Type II vitellogenesis, Aldridge, 1979). However, Taylor et al. (2004) showed that *C. atrox* in the Sonoran Desert initiate and complete vitellogenesis in the spring of a reproductive year (Type I vitellogenesis, Aldridge, 1979). Fitch (1949) asserted that *C. oreganus* in central California follow a similar pattern. This assertion was based on measurements of follicle length in spring samples. However, Fitch's data were almost identical to the spring follicle measurements of Rahn (1942) and Aldridge (1979) for a closely related rattlesnake, *C. viridis,* which exhibits type II vitellogenesis,. This similarity has led researchers to question the timing of vitellogenesis in this species (Aldridge, 2002).

Snakes displaying contrasting breeding patterns are predicted to have contrasting steroid hormone profiles (Schuett et al., 2006). Estrogens (e.g. 17β-estradiol, E2) stimulate reproductive behaviors and vitellogenesis in females (Callard, 1990). Androgens (e.g. testosterone, T, and 5ά-dihydrotestosterone, DHT) stimulate reproductive behaviors and spermatogenesis in males (Moore and Lindzey, 1992; Norris, 1997). Testosterone and DHT are elevated during the periods of spermatogenesis and mating in all pit vipers studied to date (Graham et al., 2008; Schuett et al., 1997; 2002; 2005; Taylor et al., 2004). The variation observed in the mating systems of temperate viper species makes them ideal subjects for descriptive studies investigating the roles of these hormones in free-ranging populations. Snakes demonstrating the bimodal pattern of breeding should display two peaks in the concentrations of the hormones responsible for

sexual activity (i.e., T and DHT in males). Snakes demonstrating the unimodal strategy should only show one peak in plasma concentrations of these hormones. This same logic applies to the vitellogenic patterns of female snakes. Females utilizing type I vitellogenesis should show elevated concentrations of E2 in the spring only (see Taylor et al., 2004). Type II species should have elevated levels of these hormones in the fall and spring.

In addition to E2, T, and DHT, corticosterone (B) and progesterone (P) also play important roles in vertebrate reproductive cycles. Corticosterone is elevated during reproductive events in many reptile species in order to mobilize energy stores when resources are limited (Moore and Jessop, 2003; Romero, 2002). In many viviparous taxa, Progesterone, produced by the corpus luteum and placenta, plays a role in maintaining pregnancy (Custodia-Lora, 2002). Progesterone is also produced by the adrenal glands in male and non-reproductive female snakes (e.g. garter snakes, *Thamnophis*, Highfill and Mead, 1975).The role of P in non-reproductive snakes is poorly understood.

Studies of the physiological ecology of snakes have shed light on the proximate factors mediating the events in different mating systems, including mating, spermatogenesis, and vitellogenesis (Schuett et al, 2002; 2006; Taylor et al., 2004). Inter- and intra-specific studies can further increase the understanding of adaptations in response to different ecological pressures. Intraspecific comparisons among populations can be informative when drawing conclusions about the relationship between variation in the environment and variation in physiological cycles. In addition, comparison of the relationship between hormone concentrations and reproductive events in diverse taxa can provide insight into phylogenetic influences on reproductive cycles. *Crotalus oreganus* is

a member of the western rattlesnake group (Klauber, 1972). The phylogeny of the western rattlesnake group is well established (Ashton and De Queiroz, 2001; Douglas et al., 2002; Pook et al., 2000). The western group is currently composed of seven species (Douglas et al., 2002). None of these species has been the subject of studies on hormonal regulation of reproduction.

Here we describe the relationship between steroid hormone concentrations and reproductive events in C. *oreganus* at the southern end of its range in central California using three methods: (1) quantification of steroid hormone concentrations in free-ranging snakes throughout the active season (B, DHT, and T in males, B, DHT, T, E2, and P in females), (2) observation of reproductive behaviors in free-ranging snakes, and (3) examination of the reproductive anatomy of preserved museum snakes.

2. *Methods*

2.1. Study site

This study was conducted on the northernmost portion of the Chimineas Ranch unit of the Carrizo Plain Ecological Reserve in the foothills of the Caliente Mountain range in central California (35ºN, 119ºW). Habitat is primarily oak savannah and grazed grasslands with prevalent rock outcrops.

2.2. Animals and field monitoring

Like many Crotaline snakes, *C. oreganus* has male-biased sexual size dimorphism. Male snakes in this study averaged 88.13 ± 1.9 (standard error of mean, SEM) cm in snout-vent length (SVL) and 640.8 ± 42.6 g in mass (n = 39). Female snakes had an average SVL of 73.95 ± 1.6 cm and an average mass of 338.1 ± 23.0 g (n = 19).

Both male and female *C. oreganus* reach sexual maturity by about 60 cm SVL (Diller and Wallace, 2002). In order to ensure that all samples were taken from mature individuals, only snakes greater than 60 cm SVL were included in this study.

We used a combined approach of radiotelemetry and mark-recapture to study the seasonal reproductive behavior and physiology of *C. oreganus*. Twenty snakes (10 male and 10 female) were implanted with 13-gram radiotransmitters (#SI-2T, Holohil, Carp, Ont., Canada). Male snakes were implanted in the fall of 2006 and females in the spring of 2007. Each radio-tagged snake was also marked with a 12 mm PIT tag (AVID, Norco, CA, USA), and acrylic paint was injected into the three proximal rattle segments to create a unique three-color code for each snake. Snakes that had not previously been encountered (n = 42) were also marked with PIT tags and paint.

Snakes were tracked via radiotelemetry at least once per week throughout the active season in 2007-08 (March through October), and in 2008 snakes were bled at monthly intervals when possible. Previously unencountered snakes were also bled when encountered. Blood was collected from the caudal vein using a heparinized 1-cc syringe with a 25-gauge needle within five minutes of capture. Samples were placed in 1.5 ml centrifuge tubes and were centrifuged in the lab within 24 hours of collection. This time period is sufficient to ensure that steroid hormone levels are not altered (Taylor and Schuett, 2004). Plasma samples were stored at -20° C until radioimmunoassay.

Snakes were frequently inaccessible for blood sampling because they remained underground for long periods of time, a behavior likely intensified by extremely dry conditions in 2007-08. In total, 59 blood samples were collected from 37 male snakes (28 from radio-tagged males and 31 from randomly encountered males), and 41 blood

samples were collected from 19 females (30 from radio-tagged females and 11 from randomly encountered females).

2.3. Radioimmunoassay

Concentrations of steroids were measured by standard radioimmunoassay techniques following extraction and chromatographic separation (Moore et al., 2000; Wingfield and Farner, 1975). Male and female samples were run in separate assays, and B of females was determined in a separate direct assay without chromatographic separation. For individual extraction efficiency determination, we equilibrated each sample overnight with 2,000 cpm of tritiated steroid. Each sample was extracted with 5ml of distilled dichloromethane with the dichloromethane phase removed and dried in a warm water bath, under a stream of nitrogen gas, and resuspended in 10% ethyl acetate in isooctane. To remove neutral lipids and to isolate individual steroids, all samples were transferred to diatomaceous earth (Celite, Sigma) columns for chromatographic separation. For females, P4, DHT, T, and E2 were eluted with 2 ml of 2%, 1.5 ml of 10%, 2 ml of 20%, and 2.5 ml of 40% ethyl acetate in isooctane, respectively, and saved. For males, neutral lipids and other steroids were eluted with 2 ml of isooctane and discarded. Dihydrotestosterone, T, and B were eluted with 1.5 ml of 10%, 2 ml of 20%, and 2.5 ml of 50% ethyl acetate in isooctane, respectively, and saved. After this, samples were dried in a 40° C water bath under nitrogen gas, resuspended in 600 μl phosphate buffered saline, and maintained overnight at 4° C. For the direct B assay of female samples, samples were extracted with 5ml of distilled dichloromethane and resuspended in 600 μl phosphate buffered saline, and maintained overnight at 4° C. The remainder of all assays was similar. Serial dilutions for the standard curves were performed in triplicate (range

of curves: P4, 1000-2 pg; DHT, T, and E2, 500 – 1 pg; B, 2000 - 4pg). All samples were then incubated overnight with 100 μl of antiserum (P4: P-1604, Wien Laboratories, Flanders, NJ 07876; DHT and T: T-3003, Wien Laboratories, Succasunna, NJ 07876; E2: Biogenesis, Poole, England; B: Esoterix Endocrinology, Calabasas Hills, CA 91301) and 100 μl of tritiated steroid. Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Samples were counted on a liquid scintillation counter and final concentrations corrected for individual extraction efficiency.

2.4. Behavior

Reproductive behaviors were described as consortship, courtship, combat or copulation. Consortship was defined as two snakes of the opposite sex found within one meter of each other. Courtship in rattlesnakes is characterized by chin rubbing and/or intertwining of the animals' tails. Male pit vipers engage in male to male combat to compete for females (Andren, 1986; Cartpenter, 1977). Combat was defined as two or more males elevating the anterior portion of their body and attempting to force the competitor's head towards the ground. Copulation was only recorded if cloacal penetration was observed.

2.5. Data Analysis: Male hormones

Hormone concentrations in male snakes were analyzed in two ways: by collapsing the data into seasons and by comparing mean monthly hormone concentrations. For the former analysis, we partitioned the active period into three seasons, early/spring (March-April), middle/summer (May-June), and late/fall (July-October) following Aldridge

(2002). We did this to relate seasonal trends in hormones to the timing of spermatogenesis.

The mean concentration of each hormone (B, DHT, and T) in each season was compared with ANOVA or ANCOVA (see below) using all samples collected. Some males, especially radio-tagged individuals, were represented in multiple seasons. However, the paucity of recaptures prevented the use of a repeated measures analysis (i.e., only two snakes were represented in all of the three seasons). This data analysis ("full seasonal data set") has the advantage of including all data points collected, but is problematic due to the pseudoreplication introduced by the fact that some of the samples were from the same male and therefore were not independent. To address this potential problem, repeated measures on snakes sampled in multiple seasons were discarded, reducing the sample size for statistical analysis from 58 to 39 samples. Data were discarded in the following manner in order to maintain sufficient sample sizes in the three seasons. The fewest samples were obtained in fall due to the lack of surface activity, so fall samples were preferentially selected over summer and spring samples when snakes were repeatedly measured (e.g., we included the data from a snake sampled in fall and discarded any spring or summer data from that snake). Summer had the next lowest sample size, so spring samples were thrown out in favor of summer samples when the same snake was sampled in both seasons. Although this method of throwing out data is not random, it does not bias results because samples were not selected based on hormone concentrations. By discarding data in this way, we achieved a data set that was smaller but did not suffer from pseudoreplication ("reduced seasonal data set"). The results of the analysis on the full and reduced seasonal data sets resulted in the same conclusion for

each hormone (see Results), indicating that the inclusion of pseudoreplicates did not dramatically affect the results.

Finally, the mean monthly hormone concentrations were compared using the full data set. It was not possible to throw out data to eliminate pseudoreplication in this analysis, as the sample sizes for each month would have been too low. Data used for this analysis are hereafter referred to as the "full monthly data set."

All analyses were conducted using Minitab statistical software at an alpha level of 0.05. To satisfy the assumptions of normality and homogeneity of variance, B concentrations were log-transformed, DHT concentrations were ln-transformed, and T concentrations were square root transformed. Because B and T concentrations were correlated with SVL (B: $r^2 = 0.143$, $p = 0.018$; T: $r^2 = 0.177$, $p = 0.008$), SVL was used as a covariate in the analyses. For B and T, data were analyzed with ANCOVA using a GLM with season or month as a fixed factor, SVL as a covariate, and transformed hormone concentration as the dependent variable. Dihydrotestosterone was not correlated with SVL ($r^2 = 0.056$, $p = 0.148$), and results were analyzed by one-way ANOVA. All pair-wise comparisons were run using a Tukey highest significant difference test (HSD). In addition, linear regression analysis was used to evaluate correlations among concentrations of the three hormones.

2.6 Data analysis: Female hormones

For females, there were too few samples to make monthly comparisons, or to use a reduced data set lacking pseudoreplicates. For these reasons, only full seasonal female data sets were compared. Testosterone, DHT, and P were correlated with SVL (T: $r^2 = 0.15$, $p = 0.014$; DHT: $r^2 = 0.31$, $p < 0.001$; P: $r^2 = 0.28$, $p = 0.001$) and were analyzed

using ANCOVA with SVL as the covariate. Dihydrotestosterone and P were square root transformed, and T was log-transformed to satisfy ANCOVA assumptions. 17 β-estradiol and B were not correlated with SVL and were therefore analyzed without SVL as a covariate. Corticosterone was square root transformed to meet ANOVA assumptions, and was analyzed by one way ANOVA. We were unable to meet the assumption of normality for E2 concentration data, so seasonal E2 concentrations were analyzed using a Kruskall-Wallis test.

2.7. Reproductive tissues

Reproductive tissues (testes and follicles) were examined from museum specimens (appendix 2). Four of the snakes used in this study were freshly collected for another study. Snakes in this study were collected from populations throughout California. An incision was made on the ventral surface of each snake from mid-body to the region just posterior to the gonads. For males, left testis length, width, and height were measured with digital calipers (the left testis was chosen because many of the specimens were missing the right testis, presumably removed for another study) in order to calculate left testis volume. Females were evaluated for reproductive state by measuring the length of the largest follicle present.

2.8. Data analysis: Male museum specimens

Left testis volume (LTV) was square root transformed to meet the assumptions of the ANCOVA. Because LTV was strongly correlated with SVL, data were analyzed by ANCOVA with season or month as a fixed factor, SVL as a covariate, and LTV as the dependent variable.

2.9. Data analysis: Female museum specimens

Secondary vitellogenesis was identified by the presence of enlarged (>10mm) follicles in female reproductive tracts. Rahn (1942) described follicles during primary vitellogenesis (non-reproductive) of a closely related rattlesnake species, *C. viridis*, as 4-6 mm in length. Follicles greater than 6 mm in length are likely to have entered secondary vitellogenesis (Aldridge, 1979). To err on the side of caution in diagnosing fall vitellogenesis, we considered follicles over 10 mm in length to have entered secondary vitellogenesis.

3. Results

3.1. *Seasonal behavior*

Copulation was observed once in September. Courtship behaviors were observed on two occasions in April. No combat was observed in this study. 67% of pairings were observed in April (Fig. 1). Appendix 1 lists the dates of all observed pairings.

3.2. *Male hormone data*

Mean seasonal hormone levels (T, DHT, and B) are presented in Fig. 2. Descriptive statistics are presented in Table 1. Statistical results for each hormone are discussed in the sections that follow.

3.2.1. *Testosterone*

There was a significant effect of season on T concentrations whether using the full seasonal data set ($F_{2,55} = 13.06$, $p < 0.0001$) or reduced seasonal data set ($F_{2,38} = 9.86$, $p < 0.0001$). Tukey HSD tests on both the full and reduced data sets reveal that fall T

concentrations were significantly higher than both spring and summer, which were not significantly different from each other. Full monthly analysis revealed a significant effect of month ($F_{6,50} = 5.15$, $p < 0.001$). Post hoc analyses showed that T concentrations in August were significantly higher than in April, May, and June. All other pair-wise comparisons were non-significant (Fig. 2A).

3.2.2. *Dihydrotestosterone*

There was a significant effect of season on DHT concentrations whether using the full seasonal data set ($F_{2,55} = 6.14$, $p = 0.004$) or reduced seasonal data set ($F_{2,38} = 4.32$, $p = 0.021$). Tukey HSD tests on either the full or reduced data sets show that DHT concentrations were significantly lower in summer than in fall or spring, which were not significantly different from each other. There was also an effect of month on DHT concentrations ($F = 2.47$, $p = 0.036$). However, a Tukey HSD test did not indicate any significant difference between any pair of months. Linear regression analysis showed a significant correlation between T and DHT ($r^2 = 0.56$, $p < 0.001$).

3.2.3. *Corticosterone*

Results of ANCOVA on B concentrations revealed no significant effect of season whether using the full seasonal data set ($F_{2,55} = 0.75$, $p = 0.48$) or reduced seasonal data set ($F_{2,38} = 0.71$, $p = 0.49$). The full monthly analysis also showed no significant variation ($F_{6,50} = 1.33$, $p = 0.26$). Linear regression analysis showed no correlation between B and T ($r^2 = 0.001$, $p = 0.79$) or DHT ($r^2 = 0.02$, $p = 0.30$).

3.3. *Male testis volume*

ANCOVA results indicate a significant effect of season ($F_{2,58} = 3.47$, $p = 0.038$) and month ($F_{7,54} = 2.51$, $p = 0.028$) on LTV (Fig. 3). Seasonal post hoc analysis showed

that fall LTV was significantly higher than in summer and spring, which were not significantly different from each other. Tukey HSD tests revealed that LTV in September was significantly higher than in April or May. All other pair-wise comparisons were not significant.

3.4. *Female hormones*

We did not detect any significant seasonal variation in any hormone concentration in female snakes (DHT: $F_{2,38} = 0.56$, $p = 0.58$; T: $F_{2,38} = 1.0$, $p = 0.78$; P: $F_{2,38} = 0.51$, $p = 0.61$; B: $F_{2,38} = 1.27$, $p = 0.29$; E2: $H = 1.05$, $p = 0.59$). It is important to note that these analyses likely included both reproductive and non-reproductive females, and there was large variation in E2, T, DHT, and P concentrations within each season. Results of pair-wise correlations between each hormone indicated positive correlations between T and DHT ($r = 0.7833$, $p < 0.0001$), T and E2 ($r = 0.5101$, $p < 0.0009$), T and P ($r = 0.7217$, $p < 0.0001$), and DHT and P ($r = 0.9724$, $p < 0.0001$). All other correlations were non-significant (Table 2).

3.5. *Female museum data*

Vitellogenic follicles were present in specimens collected in the months of Mar, Apr, May, June, Jul, Sep, and Oct. The presence of follicles that had entered secondary vitellogenesis in the late summer and fall is indicative of Type II vitellogenesis.

4. Discussion

Male *C. oreganus* in central California exhibit peaks in Dihydrotestosterone concentration in spring and fall, similar to other bimodally breeding pit vipers (*A. contortrix*, Schuett et al., 1997; *C. scutulatus*, Schuett et al., 2002; *C. atrox*, Taylor et al, 2004). In *C. oreganus*, T and DHT are elevated in the fall, coinciding with spermatogenesis and mating behavior (Aldridge, 2002; Fitch, 1949; Hersek et al., 1992). However, the majority of observed breeding behavior in this population occurred in spring, when T concentrations were not significantly elevated above levels quantified outside the breeding season. However, it is noteworthy that four of the seven males sampled in March had very high T concentrations (>80 ng/ml). Testosterone concentrations in this month were highly variable among individuals, and this variation led to a lack of a statistically significant difference in T concentration between spring and summer months. Highly variable T concentrations in March could have been the result of many factors, as there are numerous potential causes of variation in plasma T concentration in vertebrates, including time of day, genetic effects, and social context (reviewed in Kempenaers et al., 2008). These factors were not controlled for in this study and may have been responsible for the individual variation in T concentrations measured in spring. Interestingly, T did not approach peak concentrations in any males sampled during the peak month of breeding (April). Concentrations in April were above basal, but were significantly lower than concentrations during spermatogenesis in August. This supports the "conditioning" or "organizing" role of testosterone suggested by Crews (1991) and St. Girons et al. (1993), under which T prepares a snake for the act of mating,

but is not necessarily circulating at peak levels at the time that mating occurs. Dihydrotestosterone concentrations, on the other hand, were elevated in spring and fall, and peak concentrations coincided with the month of peak breeding behavior (April). Dihydrotestosterone is elevated during the breeding season in all pit vipers in which levels of this hormone have been measured (*C. scutulatus,* Schuett, 2002; *C. atrox,* Schuett, 2005*; C. molossus,* Schuett, 2005).

Our results for plasma B concentrations in male snakes are similar to those reported for *C. atrox* in the Sonoran Desert (Taylor et al., 2004). There was no correlation between B and androgens (T and DHT), and there was no seasonal or monthly variation in B concentrations. In many reptiles studied, B follows a seasonal cycle, with elevated concentrations occurring at the time of breeding (reviewed in Romero, 2002). The energy mobilization hypothesis (EMH) suggests that peak B levels coincide with the most energy-limited time of year. Because reproduction requires a significant investment of energy in some species, the breeding season is often the most energy-limited time of the year and thus is characterized by elevated B concentrations (Moore and Jessop, 2003; Romero, 2002). Corticosterone levels in *C. oreganus* are not elevated during either breeding season, corroborating studies by Taylor et al. (2004) and Graham et al. (2008) suggesting that male temperate pit vipers may not be subject to the energy limitations experienced by many reptile species during reproduction. Temperate pit viper species generally have low standard metabolic rates compared to most other reptiles and have large fat reserves, which may allow them to fuel the events of reproduction without initiating an adrenal response (Andrews and Pough, 1985; Taylor et al., 2004, Tinkle, 1962).

Although no significant seasonal difference in hormone concentrations was detected in females, presumably due to high variation in hormone concentrations due to inclusion of reproductive and non-reproductive females and a low sample size, 17β-estradiol, T, and DHT were highest during the spring and fall breeding seasons, which are coincident with the months in which vitellogenesis occurs in this population. 17β-estradiol directly stimulates the synthesis of vitellogenin in the reptilian liver and is most likely elevated to perform its role in vitellogenesis (Callard et al., 1990). However, E2 may also play a role in female receptivity and estrous behavior (Rhen and Crews, 2000; Whittier and Tokarz, 1992).

This study is the first to quantify DHT in addition to T concentrations in female free-ranging pit vipers. Our results indicate a possible role of T and DHT in reproductive behavior and/or vitellogenesis, as peak androgen levels were recorded during these events. However, because these events occur at the same time in this population of *C. oreganus*, it is not possible to ascertain the function of these hormones without manipulative experiments. The roles of T and DHT in the female cycle of snakes, and female vertebrates in general, are poorly understood (Staub and De Beer, 1997). Saint Girons et al. (1993) recorded elevated DHT concentrations in estrous aspic vipers (*V. aspis*), suggesting that DHT may be involved in stimulating female reproductive behavior. Testosterone is elevated during vitellogenesis in some snake species, but one must consider its role as a precursor to E2 as a confounding factor (Bona-Gallo et al., 1980; Taylor et al., 2004; Whittier et al, 1987). Dihydrotestosterone, however, is a non-aromatizable androgen, eliminating the confounding effect of a precursor-product relationship with E2. A review of the literature on the role of androgens in vertebrate

reproduction suggests that androgens indeed play a role in both the development and regulation of the female reproductive system (Staub and De Beer, 1997), but uncovering the exact role of these hormones in *C. oreganus* requires further study.

There are significant correlations between many of the steroid hormones quantified in this study. Progesterone concentrations were correlated with T and DHT concentrations. This correlation may have resulted from the fact that progesterone is an intermediate in the synthesis of androgens in vertebrates (Slaunwhite and Samuels, 1956). The correlation between DHT and P is very strong, and warrants further investigation of any functional significance of this correlation. 17β-estradiol is positively correlated with T, which could, once again, be due to a precursor-product relationship, as E2 is derived from T via aromatization.

Examination of male anatomy revealed that *C. oreganus* exhibit hypertrophy of the testes coincident with spermatogenesis in the late summer and fall. Testes are not hypertrophied during the spring breeding season. Left testis volume was at its lowest in the spring, when some males have high levels of circulating androgens and when the majority of breeding behavior was observed. This result contradicts a study (Schuett et al., 2002) on another rattlesnake with a bimodal mating system and androgen profile, *C. scutulatus*. That study found increased testis length and mass in both spring and late summer/fall coincident with elevated androgen concentrations (Schuett et al., 2002). Studies on other reptiles including snakes, turtles, and alligators have shown that elevated androgen concentrations and breeding behavior do not always coincide with testis hypertrophy. In the American alligator, *Alligator mississipiensis*, testicular mass is not related to plasma T concentrations in the late summer breeding season (Lance, 1989). In

the softshell turtle, *Trionyx sinensis*, and the red-sided gartersnake, *T. s. parietalis*, spring breeding is coincident with low or variable T concentrations and regressed testes (Camazine et al., 1980; Licht, 1982). These studies suggest that the breeding season and androgen production can be temporally separated from testis recrudescence. However, too few studies on testis size in bimodal snakes with known steroid hormone profiles are available for further evaluation of this phenomenon.

In conclusion, *C. oreganus* in central California demonstrate a bimodal breeding pattern and Type II vitellogenesis. Snakes breed and males engage in agonistic behaviors in the spring. Throughout spring the testes are regressed and DHT concentrations are elevated. Testosterone concentrations in spring are variable, with several snakes displaying extremely high T concentrations in March. In the fall season, male *C. oreganus* undergo spermatogenesis in addition to displaying breeding and agonistic behaviors (Aldridge, 2002; Hersek et al., 1992). Testosterone and DHT concentrations are elevated during this time. Corticosterone concentrations are not elevated in either breeding season, suggesting that an adrenal response is not necessary during the breeding season of this population. Reproductive female *C. oreganus* exhibit breeding behaviors and undergo vitellogenesis in the spring and late summer/fall. During these seasons, several females had high E2, T, and DHT concentrations. These hormones have all been implicated as role players in the production of breeding behavior and/or vitellogenesis in female reptiles. There was a large amount of within-season variation in the concentrations of all hormones quantified. More controlled studies are required to identify the sources of such variation. This study adds to the growing body of literature on the interplay between steroid hormones and the events of vertebrate reproduction in

wild populations. It is the first study quantifying E2, P, T, DHT and B in a free-ranging pit viper. However, this was a descriptive study and does not identify the causal factors underlying the relationships observed. Further controlled experiments are required to identify the role that these hormones play as proximate mediators of the events of the reproductive cycle.

Table 1: Mean plasma hormone levels of *Crotalus oreganus* in ng/ml (± SEM). Months where no snakes were sampled or hormones were not quantified are marked with "-."

	Sex	n	Testosterone	DHT	Corticosterone	17β-estradiol	Progesterone
Mar	Male	9	60.8 ± 14.4	1.2 ± 0.2	126.8 ± 27.4	-	-
	Female	5	0.2 ± 0.02	0.3 ± 0.03	55.7 ± 20.3	0.2 ± 0.03	0.6 ± 0.1
Apr	Male	24	31.1 ± 3.7	1.7 ± 0.4	73.0 ± 11.0	-	-
	Female	10	0.6 ± 0.1	0.7 ± 0.1	76.8 ± 19.6	10.0 ± 8.3	1.2 ± 0.1
May	Male	6	16.1 ± 4.7	0.3 ± 0.04	89.1 ± 15.0	-	-
	Female	11	0.9 ± 0.3	0.7 ± 0.03	78.8 ± 19.5	8.1 ± 6.1	1.2 ± 0.1
Jun	Male	10	20.5 ± 6.0	0.8 ± 0.3	53.4 ± 11.7	-	-
	Female	5	0.2 ± 0.03	0.3 ± 0.04	59.3 ± 39.9	0.2 ± 0.02	0.6 ± 0.04
Jul	Male	2	50.1 ± 6.6	0.8 ± 0.03	12.7 ± 5.9	-	-
	Female	2	0.2 ± 0.002	0.3 ± 0.02	50.7 ± 11.5	0.2 ± 0.1	0.7 ± 0.04
Aug	Male	4	88.1 ± 16.8	1.5 ± 0.4	67.8 ± 29.2	-	-
	Female	2	0.2 ± 0.02	0.3 ± 0.03	19.4 ± 5.0	0.2 ± 0.01	0.7 ± 0.03
Sep	Male	3	76.3 ± 12.9	1.7 ± 0.7	63.1 ± 24.2	-	-
	Female	0	-	-	-	-	-
Oct	Male	0	-	-	-	-	-
	Female	4	0.8 ± 0.5	1.2 ± 1.0	28.2 ± 12.3	17.6 ± 11.7	2.0 ± 1.4

Table 2: Correlation table for all pair-wise comparisons of female hormone concentrations in *C. oreganus*. Significant correlations are highlighted in bold.

Variable	**By Variable**	**R**	**P**
E2	DHT	0.1385	0.4003
T	**DHT**	**0.7833**	**<0.0001**
T	**E2**	**0.5101**	**0.0009**
P	**DHT**	**0.9724**	**<0.0001**
P	E2	0.1076	0.5142
P	**T**	**0.7217**	**<0.0001**
B	DHT	0.0015	0.9928
B	E2	0.113	0.4933
B	T	0.0741	0.6537
B	P	-0.045	0.7854

Figure Legends:

Figure 1: Observed pairings of male and female *Crotalus oreganus* during the 2007 and 2008 active seasons. 67% of observed pairings occured in April. Two courtships were observed in Apr, and one copulation was observed in Sep.

Figure 2: Mean monthly and seasonal plasma hormone concentrations in male *Crotalus orgeganus*. Sample sizes are in parentheses. (A) Plasma T and DHT concentrations. Plasma T concentrations were significantly higher in fall than in summer or spring. Plasma DHT concentrations were significantly higher in spring and fall than in summer. Only T concentrations were significantly different when analyzed by month. Months with significantly different T concentrations are assigned different letters. (B) Plasma B. There were no significant seasonal or monthly differences in B concentrations.

Figure 3: Square root of left testis volume (LTV) vs. snout-vent length (SVL) in each season in *Crotalus oreganus*. Fall LTV is significantly greater than both summer and spring, which were not significantly different from each other.

Figure 4: Mean monthly plasma hormone concentrations in female *Crotalus oreganus*. There were no diagnosable seasonal trends in the concentrations of these hormones. (A) Plasma E2, T, and DHT concentrations. (B) B and P concentrations.

Figure 5: Length of the largest follicle measured in female *Crotalus oreganus* museum specimens. All follicles < 5mm in length are shown as 5mm. Vitellogenesis begins in fall and continues through the following spring.

Figure 1

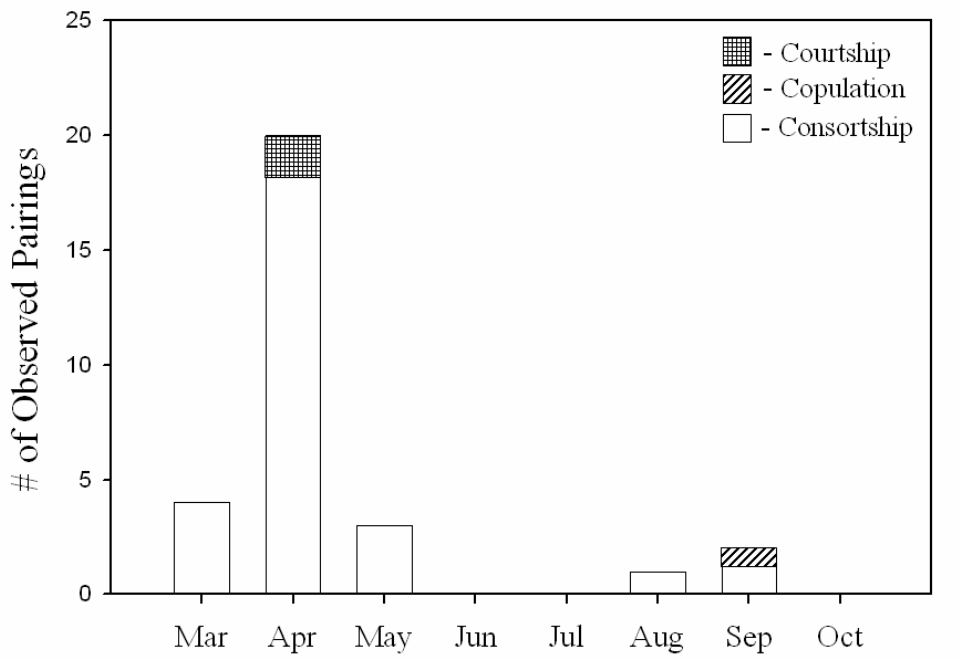
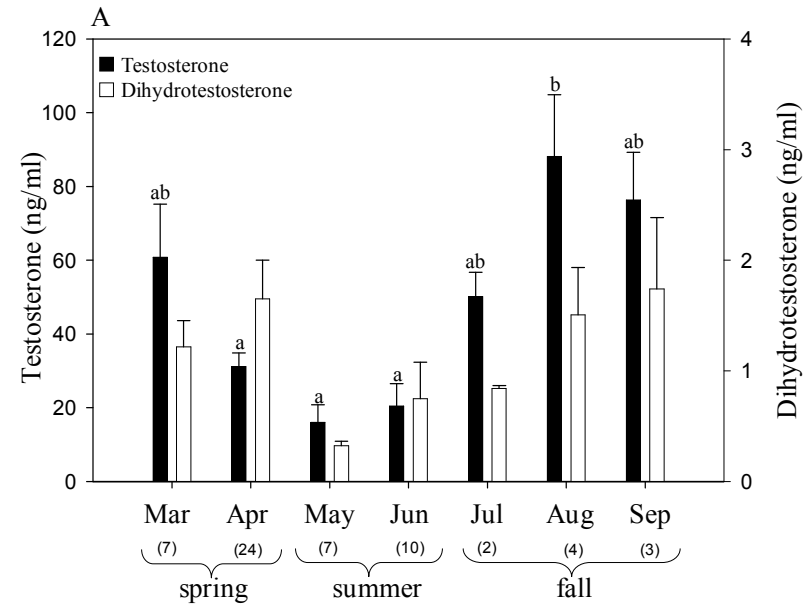

# of Observed Pairings
25
20
15
10
5
0
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
- Courtship
- Copulation
- Consortship

Figure 2


A
Testosterone
Dihydrotestosterone
Testosterone (ng/ml)
Dihydrotestosterone (ng/ml)
120
100
80
60
40
20
0
4
3
2
1
0
ab
a
a
ab
b
ab
Mar
Apr
May
Jun
Jul
Aug
Sep
(7)
(24)
(7)
(10)
(2)
(4)
(3)
spring
summer
fall

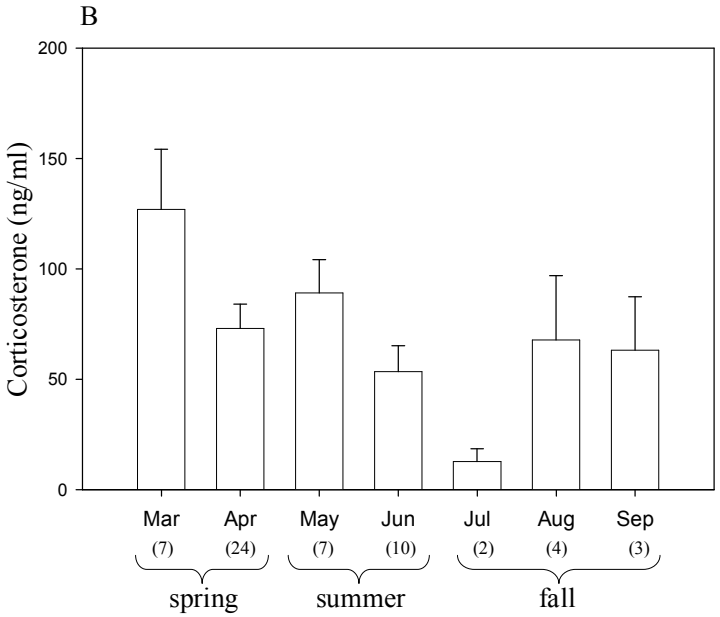

B
Corticosterone (ng/ml)
200
150
100
50
0
Mar
Apr
May
Jun
Jul
Aug
Sep
(7)
(24)
(7)
(10)
(2)
(4)
(3)
spring
summer
fall

Figure 3

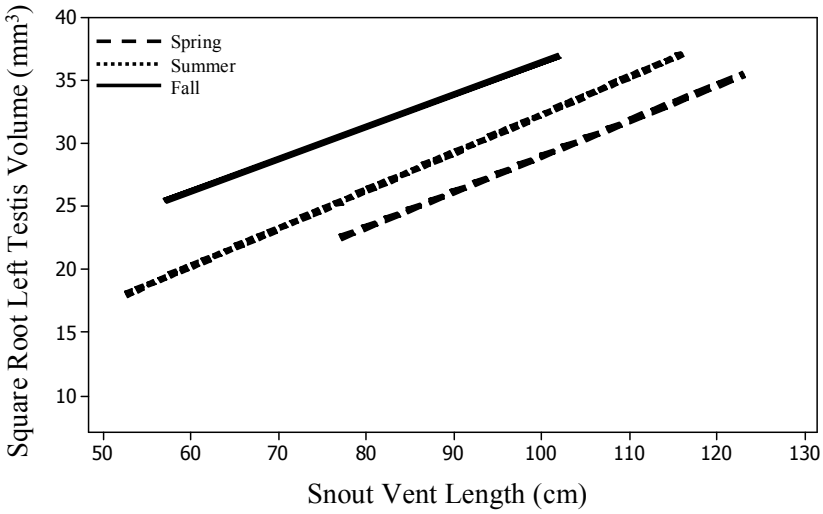

Square Root Left Testis Volume (mm3)
40
35
30
25
20
15
10
Spring
Summer
Fall
50
60
70
80
90
100
110
120
130
Snout Vent Length (cm)

Figure 4

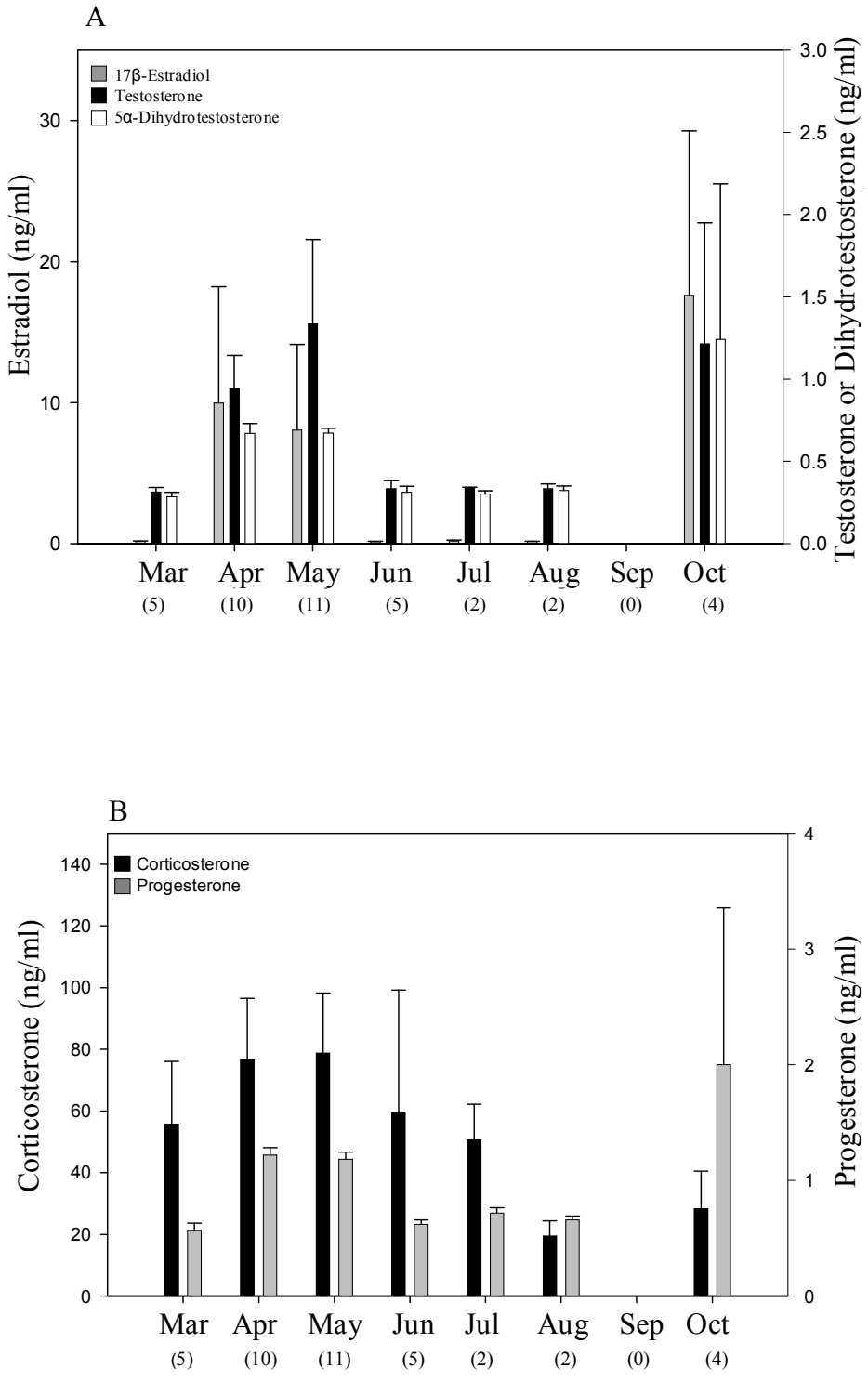
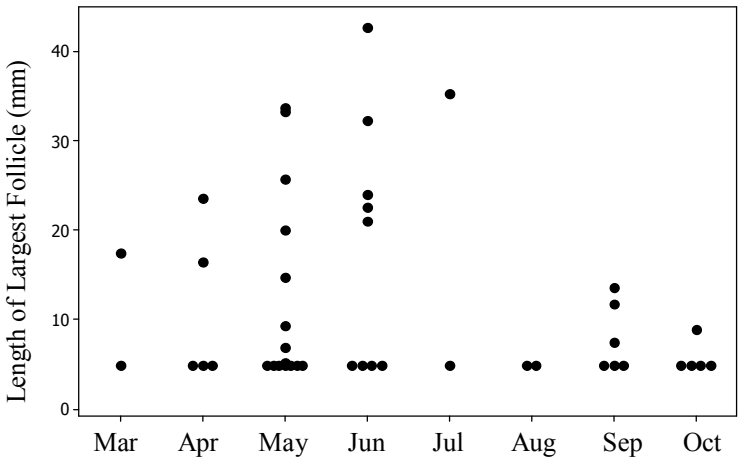

A
17β-Estradiol
Testosterone
5α-Dihydrotestosterone
Estradiol (ng/ml)
Testosterone or Dihydrotestosterone (ng/ml)
0
10
20
30
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Mar (5)
Apr (10)
May (11)
Jun (5)
Jul (2)
Aug (2)
Sep (0)
Oct (4)
B
Corticosterone
Progesterone
Corticosterone (ng/ml)
Progesterone (ng/ml)
0
20
40
60
80
100
120
140
0
1
2
3
4
Mar (5)
Apr (10)
May (11)
Jun (5)
Jul (2)
Aug (2)
Sep (0)
Oct (4)

Figure 5


Length of Largest Follicle (mm)
0
10
20
30
40
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct

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Appendix 1: Dates of all observed pairings for the 2007 and 2008 active season of *Crotalus oreganus*. Copulations and courtships are highlighted in bold. The third column, titled "Contact", indicates whether the pairing involved physical contact between the pair. The consortship marked with a * represents an occasion where a male was pulled out of a burrow with a hemipenis fully everted. A radio-tagged female was tracked to the same burrow.

Date	Behavior	Contact
3/11/2007	Consortship	No
3/24/2007	Consortship	Yes
4/6/2007	**Courtship**	Yes
4/7/2007	Consortship	No
5/4/2007	Consortship	Yes
4/7/2007	**Courtship**	Yes
4/1/2007	Consortship	Yes
3/23/2008	Consortship	Yes
3/24/2008	Consortship	No
4/5/2008	Consortship	No
4/5/2008	Consortship	Yes
4/5/2008	Consortship	Yes
4/5/2008	Consortship	No
4/5/2008	Consortship	No
4/6/2008	Consortship	Yes
4/6/2008	Consortship	No
4/12/2008	Consortship	No
4/12/2008	Consortship	Yes
4/12/2008	Consortship	Yes
4/19/2008	Consortship	No
4/19/2008	Consortship	No
4/19/2008	Consortship	Yes
4/26/2008	Consortship	No
4/26/2008	Consortship	Yes
4/26/2008	Consortship	Yes
5/2/2008	Consortship	Yes*
5/3/2008	Consortship	Yes
8/24/2008	Consortship	No
9/15/2008	Consortship	No
9/15/2008	**Copulation**	Yes

Appendix 2: List of catalogue numbers of museum specimens of *Crotalus orgeanus* examined in this study. Museum names are abbreviated as follows: Museum of Vertebrate Zoology at the University of California at Berkeley (MVZ), University of California at Santa Barbara Museum (UCSB), and Santa Barbara Natural History Museum (SBNHM). (A) Male snakes examined. (B) Female snakes examined.

MVZ: (A) 193428, 28218, 28214, 56723, 35466, 24838, 29281, 21917, 18539, 17585, 5329, 28772, 29335, 62064, 62068, 81066, 24254, 12364, 92685, 228714, 18407, 21381, 24252, 2777, 2775, 14597, 3799, 3800, 16462, 31841, 25321, 16464, 191378, 249868, 215726, 191407. (B) 57076, 58265, 2781, 41172, 24860, 229507, 170801, 249869, 179969, 215727, 191390, 191413, 191406, 24253, 21380, 21382, 83653, 17955, 16855, 62067, 193429.

UCSB: (A) 206223, 30722, 23178, 23170, 11370, 11500, 11502, 11501, 15564, 15563, 15547, 11368, 15779, 20064, 20062, 19951, 15561, 13504, 13502, 14735, 14319, 14318, 16022. (B) 2777, 2772, 34111, 147963, 192218, 79235, 17572, 18945, 64147, 56724, 44905, 6845, 6841, 56722, 19365, 12181, 14317, 15546, 13501, 14737, 14734.

SBNHM: (A) 2346. (B) 983, 910, 1351.